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by Sex Hormone-Binding Globulin

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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	12
Reportable Outcomes.....	13
Conclusions.....	13
References.....	14
Appendices.....	15

“Autocrine and Paracrine Control of Breast Cancer Growth by Sex Hormone-Binding Globulin”

INTRODUCTION

We propose that the expression of Sex Hormone-Binding Globulin (SHBG) by breast cancer cells is biologically regulated and that this SHBG functions to alter the effects of estrogens within the breast cancer cell. We have shown that the plasma protein sex hormone-binding globulin (SHBG) not only binds estrogens in plasma, but also is part of an estrogen signal transduction system that starts with a receptor (R_{SHBG}) for SHBG on breast cell membranes (1). The SHBG- R_{SHBG} complex is activated by an appropriate steroid hormone, such as estradiol (E_2), (forming the new complex, E_2 -SHBG- R_{SHBG}), to trigger a second messenger system to produce cAMP within minutes after the steroid binds. We have shown that frozen sections of normal and cancerous breast cells stain with anti-SHBG antibodies and that these same cells contain SHBG mRNA. Further, the well-known breast cancer cell line, MCF-7, contains both SHBG mRNA and SHBG protein. The expression of SHBG by breast cancer cells raises the important question of how the local regulation of SHBG synthesis and secretion affects both the sequestration of steroid hormones within the breast, and estrogen induced signal transduction at the cell membrane. The local synthesis of SHBG is consistent with an autocrine/paracrine role for this protein in breast cancer.

To this end, we planned studies to conduct genetically and pharmacologically based studies to address our hypothesis. These included: 1) generation and characterization of breast cell lines that inducibly or constitutively overexpress SHBG and condition their medium with secreted SHBG; 2) characterization of SHBG mRNA expression in breast cell lines; 3) characterization of SHBG protein expression in breast cell lines, and normal and tumor tissues. The cell lines generated will be useful for investigating the effects of SHBG expression on selective stimulation of either the estrogen receptor (ER) or the SHBG-receptor based pathways, and the effects of conditioned medium from these cell lines on cell growth.. We also have characterized SHBG gene expression in MCF-7 cells and other cell lines and tissues, generating a profile that is much more complex than previously reported. The human SHBG gene contains at least three different promoters, and the transcripts from these three promoters undergo alternative splicing of exons 4, 6, and 7. It was important to note that the secreted form of SHBG was absent in MCF-7 cells, causing us to reevaluate our original hypothesis [e.g. that SHBG exerted paracrine effects in the breast], and focus on the characterization of novel SHBG gene transcripts in breast cancer cell lines and breast tissue. These results compel further study of the biologic properties of novel SHBG isoforms expressed in the breast and how their differential expression contributes to malignant transformation.

BODY

1. Generation of inducible SHBG plasmids. We cloned the human SHBG cDNA into the inducible expression vector, pINDHyg, in both the sense (pIND/Hygro/SHBGsense) and antisense (pIND/Hygro/SHBGantisense) directions, and confirmed the fidelity of the

inserts by sequence analysis. Importantly, we decided that it would be scientifically prudent to establish intermediate MCF-7 and MDA-MB-231 cell lines that incorporated functional pVgRXR sequences so that all derivative cell lines in these studies would be from identical subclones. In this way, we minimize artifacts due to positional effects on pVgRXR genomic insertion points or clonal variance. These cell lines will also be valuable as tools for inducible expression of other proteins.

2. Generation of a nonleaky intermediate inducible MCF-7 cell line. The plasmid, pVgRXR was transfected into MCF7 cells. Stable transfectant colonies were generated by growing the transfected cells in the presence of zeocin. Twenty-four candidate zeocin resistant colonies were isolated and expanded as intermediate clonal cell lines. In transient transfection experiments with pIND/Hygro/LacZ, all clones were tested for their ability to induce expression of the LacZ gene following treatment with the inducing agent, Ponasterone A (Pon A). Three intermediate clones, MCF7-pVgRXR4, 12, and 13, demonstrated inducible LacZ activity (β -galactosidase assay). The MCF7-pVgRXR 13 cell line showed no background and had the highest degree of induction; therefore, we chose it as our intermediate cell line.

3. Generation of M13S14, an MCF-7 cell line with inducible SHBG expression, and M13V7, a vector control cell line.

We stably transfected the inducible plasmids pIND/Hygro/SHBGsense and pIND/Hygro/SHBGantisense, and the control plasmid, pIND/Hygro into MCF7-pVgRXR13 cells. Selection for incorporation of plasmid sequences was in hygromycin B. We isolated and expanded 24 colonies from each of the pIND/Hygro/SHBGsense, pIND/Hygro/SHBGantisense, and pIND/Hygro transfectants.

We tested the positive control, MCF7-pVgRXR13-pIND/Hygro/LacZ, pool of cells with Pon A (10uM), and found that approximately 8% of the induced cells turned blue in a β -galactosidase assay, while no uninduced cells from the same pool turned blue. This indicated that, not only was the transfection process successful, but more importantly that our overall strategy was feasible.

After expansion of the 24 clones from each of the other three transfections, DNA was prepared, and analyzed by PCR for uptake of SHBG sequences (or vector sequences for the control transfectants). Positive pIND/Hygro/SHBGsense clones were analyzed for induced SHBG expression by treating cells with 10 μ M ponasterone A, and measuring cellular SHBG. PonA markedly induces SHBG in M13S14 and less markedly in M13S24, which also shows some basal leakiness (**Figure 1**). As expected, PonA did not induce SHBG expression in the vector control cell line, M13V7.

We did not pursue further analysis of the antisense candidates past the PCR analysis step described above because recent work has shown that RNA interference technology provides a more prudent way to silence endogenous gene expression.

Because the inducible overexpression of SHBG worked in MCF-7 cells, we performed parallel experiments in MDA-MB-231 cells. pVgRXR was transfected into MDA-MB-

231 cells, and 24 zeomycin resistant colonies were expanded as cell lines. Unexpectedly, MDA-MB-231 cells were highly resistant to killing by the drug zeocin, including relatively large doses of up to 2 mg/ml. Because we select for pVgRXR uptake with zeocin, we instead attempted to introduce pVgRXR into MDA-MB-231 cells by cotransfecting this plasmid with the vector pCMVFlag (described below), which contains a selectable neomycin resistance gene. Transfections were performed using a 10:1 ratio of pVgRXR to pCMVFlag. We performed PCR analysis on 24 G 418 resistant colonies, and these all proved negative for pVgRXR, as did the pool of remaining colonies. We repeated transfections using even higher ratios of pVgRXR to pCMVFlag and obtained 24 candidate colonies for uptake of functional pVgRXR sequences. Unfortunately, none of these 24 colonies proved to be inducible. We therefore postponed these studies and shifted our efforts to evaluating the novel SHBG expression pattern that we discovered as part of this study (see below).

4. Anti-human SHBG antibodies for Western blot analysis.

A major technical problem that we addressed was identifying an antibody that would be suitable for Western blot analysis of cellular extracts. First, we tested all the polyclonal and monoclonal antibodies that we have generated or obtained from others using HepG2 cell extracts and purified SHBG as test substances. None proved useful for Western blot analysis. We next had custom polyclonal antisera generated against both the secreted form of SHBG (encoded by the cDNA used in these studies) and the alternative form of SHBG first described in the testis (2). Unique peptides, CLRPVLPTQSA and CFSRLRLTHPPRTW, corresponding to the respective SHBG isoforms, were synthesized and used to immunize rabbits. Affinity purified antisera were positive by ELISA assay, however they did not prove useful for Western blot analysis. Fortunately, at the beginning of 2004, we obtained a polyclonal antibody, WAK-S102-12-53 (WAK-Chemie, Steinbach, Germany), which is useful for Western blot analysis. As detailed below, we used WAK-S102-12-53 to confirm ELISA and PCR results on cell lines that inducibly or constitutively express various SHBG constructs at elevated levels.

5. Generation of pSHBG-FL and pSHBG-MP, plasmid constructs that direct constitutive expression of SHBG. We synthesized two additional constructs for evaluating the effects of constitutively expressed SHBG in MDA-MB-231 cells. The vector used for these experiments was pCMVFlag, a plasmid containing a CMV promoter upstream of an ATG start codon, immediately followed by three iterations of a Flag tag (4) sequence. We generated: 1) pSHBG-FL, a plasmid containing the full length SHBG cDNA sequence cloned immediately downstream of the Flag tags and in the same reading frame, and 2) pSHBG-MP, a plasmid containing SHBG cDNA lacking the 29 amino acid amino terminal leader sequence which, in the liver, is cleaved from the nascent protein before secretion. Thus, this Flag tagged protein mimics the mature, processed SHBG found in serum. However, since it lacks the leader sequence, we hypothesized that the SHBG-MP protein would remain inside cells. We expected that the Flag tag would allow us to specifically detect the expressed pSHBG-FL and pSHBG-MP proteins in western blots. In addition, the Flag tag would produce different sized SHBG proteins, distinguishable from endogenous SHBG.

We expected that SHBG-FL might be detectable in cells, but it would have the Flag tag cleaved along with the signal peptide prior to secretion. However, if the cleavage system was overloaded with SHBG-FL protein, we might also detect residual full length SHBG-FL protein. We expected that the SHBG-MP construct would remain inside cells because it lacked a leader sequence, and might serve to mimic the fate of SHBG absorbed from outside the cell. The CMV promoter would direct constitutive expression of these proteins at elevated levels.

6. Generation of MCF-7 and MDA-MB-231 clonal cell lines that constitutively overexpress the pSHBG-FL and pSHBG-MP constructs. pSHBG-FL and pSHBG-MP were transfected into MDA-MB-231 cells. Following selection, 12 resistant colonies from each transfection were isolated and expanded. MDA-MB-231-MP7 was positive in Western blot analysis using the anti-Flag monoclonal antibody (**Figure 2A**), and cell extracts from 12 MDA-MB-231-FL clones have recently been prepared for analysis.

We also transfected pSHBG-FL and pSHBG-MP into MCF-7 cells, and expanded 12 individual clones from each transfection. Three MCF-7-FL clones had elevated amounts of SHBG protein (ELISA). Western blot analysis showed that MCF-7-FL10 had high expression of the Flag-tagged SHBG construct (**Figure 2B**). Furthermore, this clone secreted non Flag-tagged SHBG (**Figure 2B**), demonstrating that the leader peptide is properly processed from Flag-tagged SHBG-FL, and that the mature processed protein is secreted just like the secreted form of SHBG in the liver. We isolated 12 MCF-7-MP clones, and Western blot analysis showed that MCF-7-MP11 had high expression of the Flag-tagged SHBG-MP construct.

7. Construction of pSHBG-myc, a plasmid directing constitutive expression of carboxyl terminal myc-tagged SHBG. The SHBG-FL, amino terminal Flag-tagged protein, loses its Flag-tag upon cleavage of the amino terminal SHBG signal peptide prior to secretion. To obtain a secreted tagged-SHBG species that could be immunologically distinguishable from endogenously synthesized SHBG in MCF-7 cells, we synthesized pSHBG-myc. pSHBG-myc encodes a full length SHBG protein whose expression is driven by a constitutively active CMV promoter. The SHBG protein is tagged at its carboxyl terminus with a highly immunogenic peptide sequence derived from the human myc protein. Highly versatile antibodies directed against this myc tag are commercially available making it a valuable reagent for studying SHBG in breast cells.

To make this construct, we used the Xi-Clone system from Gene Therapy Systems (San Diego, Ca.). This system affords sequence specific cloning by taking advantage of the homologous recombination properties of a proprietary *E. coli* strain, therefore bypassing the need for a cloning strategy dependent on restriction sites. We designed SHBG specific PCR primers with Xi-Clone recombination sequences at their 5' ends. The forward primer had a run of amino terminal SHBG cDNA sequences following the recombination sequences. The reverse primer was longer and more complex. It contained Xi-clone recombination sequences, a stretch encoding the myc peptide, and then carboxyl terminal SHBG sequences. The forward and reverse Xi-Clone/SHBG primers were used to amplify SHBG cDNA sequences. The PCR product was purified to

remove unincorporated primers, and then was mixed with the linearized Xi-clone vector. These DNAs underwent homologous recombination following the transformation of the proprietary *E. coli* strain. Successful recombinant plasmids afforded kanamycin resistance. Over 30 kanamycin resistant colonies were isolated and plasmid DNAs were prepared and sequenced. pSHBG-myc23 contained proper sequences (often, mutations were found within the long 3' primer sequence, likely due to errors incorporated during synthesis of the primer), as was used for transfection into mammalian cells.

8. Generation of MCF-7 and HepG2 clonal cell lines that constitutively overexpress the pSHBG-myc23. MCF-7 and HepG2 cells were transfected with pSHBG-myc23, and pools of G418 colonies were obtained. The pools were screened by ELISA assay for secreted SHBG, and by Western blot for secreted and intracellular SHBG using both anti SHBG and anti-myc antibodies. Both MCF-7 and HepG2 pools were positive in all cases. To obtain clones that expressed high amounts of the pSHBG-myc23 construct, and that were pure populations of cells, the MCF-7 and HepG2 pools were plated at clonal density in 225mm cell culture dishes. From each dish, 24 individual colonies were isolated and expanded as cell lines. Cell lines were screened by immunostaining to identify those that expressed the SHBG-myc protein at high levels in all cells. We successfully identified MCF-7 SHBG-myc23-2 and HepG2 SHBG-myc23-13 as pure populations of cells that highly express the SHBG-myc construct. **Figure 3** shows the immunofluorescence of SHBG in MCF-7 SHBG-myc23-2 cells. These cells appear to have strong staining in the Golgi, as would be expected of secreted proteins, and also staining along what appears to be microfilaments. Further study of the staining pattern of these overexpressers is in progress. The successful generation of these cell lines will now allow us to use them as powerful reagents for examining SHBG uptake by MCF-7 cells, and for investigating proteins that interact with SHBG in MCF-7 cells.

9. MCF-7 cells express novel SHBG isoforms generated through alternative splicing of exons 4, 6, and 7.

The bulk of published data on SHBG gene expression at the mRNA level are based on work that was performed prior to the advent of PCR. It had been thought from early cDNA cloning experiments that two mRNA species are synthesized from the human SHBG gene (2-3). The first is the secreted form of SHBG, made in the liver. This 8 exon-long species is the isoform we have overexpressed above. The second SHBG isoform was originally described in the testis, and apart from a recent publication that shows it to be expressed in human sperm (5), it remains very poorly characterized. This isoform uses an upstream promoter and therefore has different first exon sequences. Early cDNA studies suggested that this isoform has exon 7 sequences spliced out.

We obtained data suggesting that SHBG gene expression in MCF-7 cells is different from that described above. We prepared first strand cDNA from total cellular MCF-7 RNA using oligo dT primers. Using primers that amplified exon 5-8 sequences, we unexpectedly generated three RT-PCR transcripts (**Figure 4**). Sequence analysis revealed that the largest transcript contained faithful splicing of exon 5,6,7,and 8 sequences. The intermediate transcript was missing all of exon 7 sequences. The smallest transcript was missing both exon 6 and 7 sequences.

We were unable to generate RT-PCR transcripts from MCF-7 cells using secreted SHBG exon 1 specific primers (**Figure 5**). As expected, HepG2 RT-PCR samples were positive for the secreted SHBG exon 1 transcript (6).

Next, we performed RT-PCR experiments using primers specific for SHBG exons 2 and 8. Because the SHBG gene had been reported to use two promoters, this should give us a composite picture of expression of the secreted and testis SHBG mRNA. As we later discovered, the SHBG gene contains a third, novel upstream promoter (see below). All evidence to date points to splicing of all different first exon species directly to exon 2, and termination occurring at exon 8. Therefore, the exon 2-8 RT-PCR amplification is a composite of SHBG expression derived from at least three promoters. **Figure 6, panel A** is an RT-PCR experiment using as template, first strand cDNA from HepG2, LNCaP, and MCF-7 cells, testis tissue, and an MCF-7 transfectant that constitutively overexpresses the full length secreted SHBG cDNA (MCF-7 FL-10 cells, see above). We identified, and confirmed by DNA sequence analysis, SHBG transcripts containing contiguous exon 2-8 sequences, as well as those lacking exon 7, 6 and 7, and 4, 6, and 7 sequences, respectively in HepG2, LNCaP, and MCF-7 cells, and in testis tissue. Only the full length contiguous exon 2-8 species is seen in the MCF-7 transfectant, because of the overwhelming abundance of the overexpressed full length SHBG construct. We are currently characterizing the additional bands present in the HepG2, LNCaP, and MCF-7 cells, and testis tissue lanes.

Next, we examined the expression pattern of exon 1 specific transcripts using exon 1 primers specific for (**Figure 6, panel B**) the secreted form of SHBG and (**Figure 6 Panel C**) the testis form of SHBG. HepG2 cells and normal liver express the contiguous exon 1-8 transcript, as well as those lacking exon 7, 6 and 7, and 4, 6, and 7 (very faint). Testis does not. The additional bands are currently undergoing characterization. The contiguous testis exon 1-8 transcript, as well as transcripts lacking exons 7, 6 and 7, and 4, 6, and 7 are present in LNCaP, MCF-7, and testis tissue. HepG2 mostly expresses the alternatively spliced testis transcript lacking exons 4, 6, and 7.

10. Discovery of a novel third human SHBG gene promoter.

We made an important and unexpected discovery that a novel, third human SHBG gene promoter exists, and is being used by MCF-7 and LNCaP cells. The 5' ends of SHBG gene transcripts were specifically amplified from LNCaP, MCF-7, and HepG2 cells by RT-PCR, through use of the FirstChoice RLM-RACE Kit (Ambion). Briefly, this kit invokes a series of enzymatic steps designed to allow only full length, capped RNA species to undergo ligation to a synthetic RNA adaptor of known sequence. cDNA synthesis is directed by a gene-specific oligonucleotide primer, and subsequent RT-PCR analysis is performed using an upstream adaptor-specific primer, and a nested downstream gene specific primer. In these experiments, an exon 3 specific oligo was used to direct cDNA synthesis for each of the cell lines, and RT-PCR analysis was performed using a primer set that included a nested downstream exon 2 specific primer. The resultant RT-PCR fragments (not shown) were sequenced, and consisted of unique 5'

sequences spliced directly to exon 2 sequences. The 5' sequences were mapped upstream of the prior two reported SHBG gene first exon sequences.

Figure 6, panel D is an RT-PCR experiment performed using a first exon primer specific for the novel upstream first exon sequences, and an exon 8 specific reverse primer. LNCaP cells express the novel exon 1-8 transcript, as well as transcripts lacking exons 7, 6 and 7, and 4, 6, and 7. MCF-7 cells express the transcript lacking exons 6 and 7.

6. Immunohistochemical analysis of SHBG protein expression in human breast cell lines and effects of steroid binding to SHBG on membrane localization.

SHBG is visualized clearly in the cytoplasm, but not the nucleus of MCF-7, MDA-MB-231, and 184-B5 cell lines (**Figure 7**). Note that most of the heavy staining in MCF-7 cells is perinuclear (**Figure 7A**). MCF-7 cells stain more intensely than the other lines and also (less apparent) show staining in a greater fraction of cells than do MDA-MB-231 or 184-B5 cells (**Figure 7B and 7C**). Nonpermeabilized MCF-7 cells showed membrane staining for SHBG, consistent with secretion of SHBG followed by binding to R_{SHBG} (**Figure 8A,B**). How these observations fit with our finding that alternative SHBG transcripts are synthesized in MCF-7 cells is unclear at the moment, but is consistent with a protein that retains its membrane binding site. MDA-MB-231 cells showed little if any membrane binding in nonpermeabilized cells (data not shown).

The current model for steroid signaling through R_{SHBG} includes binding of steroid-free SHBG to R_{SHBG} as an initial step. Biochemical studies have shown that SHBG, prebound to steroid, is unable to bind to R_{SHBG} . To test this model in vivo using MCF-7 cells (known to possess R_{SHBG}), we asked whether specific steroids that bind to SHBG could displace it from the membrane (7). MCF-7 cells treated with purified SHBG showed a marked increase in membrane staining, and in intracellular SHBG (**Figure 8C**). Cells treated with SHBG that had been preincubated with 2-methoxyestradiol (2MeOE₂), a steroid that binds tightly to SHBG, showed a marked decrease in membrane staining (**Figure 8D**). These results support our observations using biochemical approaches (8). We then tested the effects of MCF-7 cells treated with SHBG that had been preincubated with dihydrotestosterone (DHT) or testosterone (T) (both of which are tightly bound to SHBG) (**Figure 8E and 8F**). None of the steroids added in conjunction with SHBG appeared to have any effect on intracellular SHBG (permeabilized cells) (data not shown).

Although MDA-MB-231 cells have cytoplasmic SHBG, none is associated with the cell membrane, either from endogenous SHBG or added SHBG. These data indicated an absence of R_{SHBG} in these cells (Data not shown).

7. Immunohistochemical analysis of SHBG and in situ analysis of SHBG mRNA expression in normal human breast tissue and breast tumors.

In normal breast tissue, staining for SHBG is most intense in epithelial cells, predominating in luminal epithelial cells (**Figure 9A**). Because there is a substantial concentration of SHBG in plasma, the staining seen in sections of human breast could result from simple diffusion of SHBG from plasma with subsequent cellular uptake. To address this issue, we examined sections for SHBG mRNA expression by *in situ*

hybridization. SHBG mRNA (**Figure 10A**) is detected and is located predominantly in luminal epithelial cells. Although SHBG protein and mRNA appear to colocalize in cells, normal breast tissue is heterogeneous, with areas of intense staining and areas of zero to light staining.

SHBG protein expression is abundant in cancerous areas of the breast (**Figure 9B**) with strong staining seen in periglandular epithelium. Epithelial cells, both normal and cancerous, stain strongly for SHBG mRNA, and there is minimal staining in stromal cells (**Figure 10B**). These observations are consistent with a model in which SHBG is synthesized and secreted primarily by epithelial cells and binds to R_{SHBG} on epithelial and/or stromal cells.

We delayed our functional analysis of the effects of SHBG expression in breast cells on steroid signaling because of our novel findings that MCF-7 cells display complex expression of the SHBG gene. MCF-7 cells do not appear to make the mRNA encoding the secreted isoform that we proposed as an integral part of this study. Though we made inducible and constitutively active SHBG constructs that were successfully introduced and expressed in MCF-7 cells, our novel results suggest this does not reflect the physiologic state of SHBG expression in the human breast. Therefore, despite the availability of these cell lines, we have focused on fully characterizing SHBG gene expression in MCF-7 cells and in the breast at the mRNA level. The inducible and constitutive cell lines that we have produced for this study will be highly useful for modeling experiments on SHBG binding to R_{SHBG} and internalization of plasma SHBG. These cell lines secrete SHBG into the media at high concentrations (over 200 nM, ELISA results, data not shown).

Our immunohistochemistry data showing SHBG staining in normal breast, breast tumor tissue, and breast cancer cell lines begs the question of what SHBG isoform is being recognized. We know from staining of MCF-7 cells that they are expressing their own SHBG. Our current RT-PCR data suggests that this is not the secreted form of SHBG, but instead might be one of the alternatively spliced testis isoforms or perhaps the novel isoform originating from the upstream promoter. Although in breast tissue we detect both SHBG mRNA and protein, it appears that we need to modify our original hypothesis that the secreted form of SHBG is being synthesized endogenously. We are currently addressing the question of which SHBG isoform is being detected by our antibodies in these experiments. If it is one or more of the novel SHBG isoforms, we will address its biology. If it is the secreted form of SHBG, then we have to address the possibility that breast tissue actively internalizes SHBG from plasma.

Our results from this study have painted a new picture of extrahepatic expression of the SHBG gene. Before, it was assumed that two major SHBG transcripts existed, that which encodes the secreted form of SHBG and that which encodes the exon 7 lacking testis form. We now know that there are at least 12 different SHBG transcripts made by human cells that result from alternative splicing and alternative promoter utilization. We have shown that our system of overexpression works, and we are now set up to perform similar experiments to address the functions of these additional SHBG gene transcripts.

We expect that because these novel, alternatively spliced transcripts lack certain structural domains, they may not bind steroid, dimerize, or be secreted. It is important to note, however, that they do retain reported R_{SHBG} binding domain sequences. Our future studies and grant applications will address these biologic questions. Furthermore, because the SHBG gene lies in a hotspot for chromosomal deletion in breast cancer cells, we will address how decreased expression might be involved with cancer progression.

KEY RESEARCH ACCOMPLISHMENTS

- Generation of plasmid constructs encoding the full length human SHBG cDNA, in the sense and antisense orientations
- Performed immunohistochemical and in situ analyses of SHBG protein and mRNA expression in breast cell lines, normal breast tissue, and breast tumors.
- Generation of the intermediate cell line MCF7-pVgRXR 13, which has been stably transfected with the plasmid, pVgRXR. This cell line shows no leakiness and has a high degree of induction when transiently transfected with the β -galactosidase reporter plasmid, pIND/Hygro/LacZ.
- Identification of the anti-human SHBG polyclonal antibody, WAK-S102-12-53, for use in Western blot analysis.
- Generation of M13S14, an MCF-7 subclone engineered to inducibly express the hepatic, secreted form of SHBG. Generation of M13V7, a control MCF-7 subclone.
- Synthesis of pSHBG-FL and pSHBG-MP, flag tagged full length and mature processed SHBG constructs for constitutive expression. Generation of 12 candidate MDA-MB-231-FL clonal cell lines, and MDA-MB-231-MP7 that constitutively expresses a stably incorporated flag tagged mature processed SHBG construct. Generation of MCF-7-FL10 which constitutively expresses a stably incorporated flag tagged full length SHBG. Generation of MCF-7-MP11 which constitutively expresses a stably incorporated flag tagged mature processed SHBG construct.
- Demonstration that elevated amounts of SHBG can be made in MCF-7 and MDA-MB-231 cells, and that MCF-7 overexpressing cells secrete SHBG. Demonstration that the flag tagged mature processed SHBG protein is stable and remains intracellular.
- Synthesis of pSHBG-myc, a carboxyl terminal myc-tagged SHBG construct.
- Generation of MCF-7-SHBG-myc23-2 and HepG2-SHBG-myc23-13, clonal cell lines that express and secrete myc-tagged SHBG.
- MCF-7 cells express multiple SHBG mRNA species, including forms that retain exons 2-8, and those that splice out exons 7, 6+7, and 4, 6, and 7. RT-PCR results suggest MCF-7 cells express novel SHBG isoforms that lack the exon 1 sequences present in hepatically derived SHBG.
- MCF-7 cells use the testis promoter, and a novel upstream promoter for SHBG gene expression.

- SHBG protein is expressed in the cytoplasm of the MCF-7, MDA-MB-231, and 184-B5 cell lines. In MCF-7 cells, SHBG protein is strikingly perinuclear.
- Immunohistochemical results demonstrate SHBG binding to the membrane of MCF-7 cells, but not MDA-MB-231 cells, suggesting the latter lack R_{SHBG}. MCF-7 membrane binding can be displaced by preincubation of SHBG with 2-methoxyestradiol, T, and DHT.
- In normal breast tissue, staining for SHBG is most intense in epithelial cells, predominating in luminal epithelial cells. SHBG mRNA colocalizes to these same cells, suggesting that in vivo, endogenously synthesized SHBG mRNA is translated and expressed. Normal breast tissue itself appears heterogeneous, with areas of intense staining and areas of zero to light staining.
- SHBG protein expression is abundant in cancerous areas of the breast with strong staining seen in periglandular epithelia. Strong staining for the SHBG mRNA was seen in epithelial cells as well as in carcinoma cells infiltrating the stroma adjacent to the epithelial cells.

REPORTABLE OUTCOMES:

1. Fourth International Symposium on Hormonal Carcinogenesis. Valencia, Spain, June 21-25, 2003

Poster presentation and book chapter:

IMMUNOHISTOCHEMICAL AND *IN SITU* DETECTION OF SEX HORMONE-BINDING GLOBULIN (SHBG) EXPRESSION IN BREAST AND PROSTATE CANCER: IMPLICATIONS FOR HORMONE REGULATION

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1. Third International Meeting- Rapid Responses to Steroid Hormones. Florence, Italy, Sept. 12-14, 2003

Poster presentation:

IMMUNOHISTOCHEMICAL AND *IN SITU* DETECTION OF SEX HORMONE-BINDING GLOBULIN (SHBG) EXPRESSION IN BREAST AND PROSTATE CANCER: IMPLICATIONS FOR HORMONE REGULATION

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CONCLUSIONS:

We have shown that MCF-7 and MDA-MB-231 cells can be used to produce the hepatically secreted isoform of SHBG. From our initial RT-PCR results, we expected that this was the major isoform expressed in breast cells. However, based on our newer RT-PCR results, this is not the case. Alternative splicing and alternative promoter

utilization results in the expression of mRNAs encoding potentially novel SHBG isoform(s) in MCF-7 cells. If this is also the case in normal breast epithelial cells in general, this could change our prior hypothesis of how allelic deletions of the SHBG gene locus could contribute to breast cancer. The distinct perinuclear immunohistochemical staining of SHBG seen in MCF-7 cells is striking, and raises new questions about SHBG function.

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APPENDICES:

Figure 1. SHBG overexpression is induced by PonA in M13S14 cells

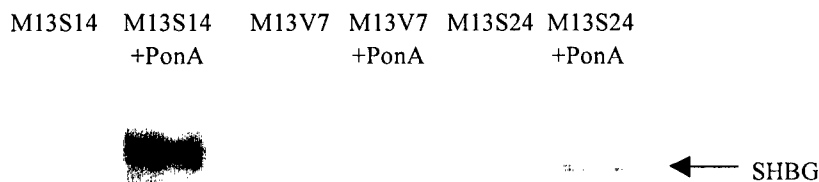


Figure 1: PonA induces SHBG protein expression in MCF-7 cells stably transfected with pVgRXR (a plasmid encoding the PonA-activatable transactivator), and the inducible construct, pIND/Hygro/SHBGsense (a plasmid that expresses the secreted form of SHBG in response to the activated transactivator). M13S14, M13V7, and M13S24 cells were plated in duplicate in 6 well dishes at 75% confluence. After incubating for 48 hours, cells in one well were exposed to 10uM PonA for 24 hours, while control, unexposed cells were mock treated with solvent. Total cellular protein was prepared and analyzed by Western blot. Low amounts of SHBG were visible on the original film in the M13V7 lanes. Parental M13 cells showed similarly low expression of endogenous SHBG, on a par with untreated M13V7 cells, and were not affected by PonA treatment

Figure 2. Western blot analysis of SHBG overexpression in MB-MDA-231 and MCF-7 clonal cell lines

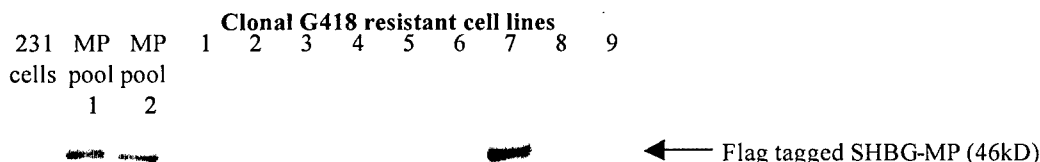


Figure 2A: MB-MDA-231-MP7 cells (MB -MDA-231cells stably transfected with pSHBG-MP, a construct that encodes a Flag-tagged, mature, processed form of SHBG) constitutively overexpress Flag-tagged SHBG-MP. Total cellular protein extracts were prepared from two MB-MDA-231 transfectant pools and candidate MB-MDA-231-MP clonal cell lines. Western blot analysis, shown above, was used to detect Flag tagged SHBG-MP expression using the Anti-Flag M2 monoclonal antibody (Sigma).

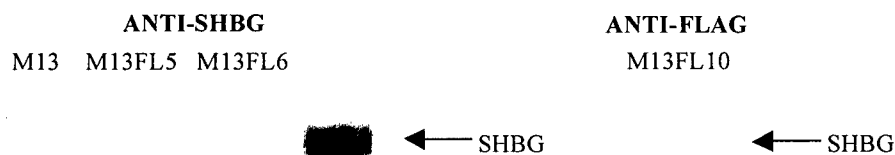


Figure 2B: M13FL10 (MB -MDA-231 cells stably transfected with pSHBG-FL, a construct that encodes a Flag tagged, full length secreted form of SHBG) cells secrete SHBG. M13, M13FL5, M13FL6, and M13FL10 cells were grown in 6 well dishes for 4 days in 1ml of serum-free medium. Cells appeared viable after this period of time. Conditioned medium was collected and centrifuged to remove cells and cellular debris. 30ul aliquots of conditioned medium were analyzed by Western blot, left, using the anti-human SHBG WAK-S102-12-53 antibody. Right, Western blot analysis of M13FL10 using the Anti-Flag M2 monoclonal antibody (no band was apparent). Sample on the right was from the same original Western blot shown as in Figure 2A.

Figure 3. Expression of SHBG-myc in MCF-7-SHBG-myc23-2 cells

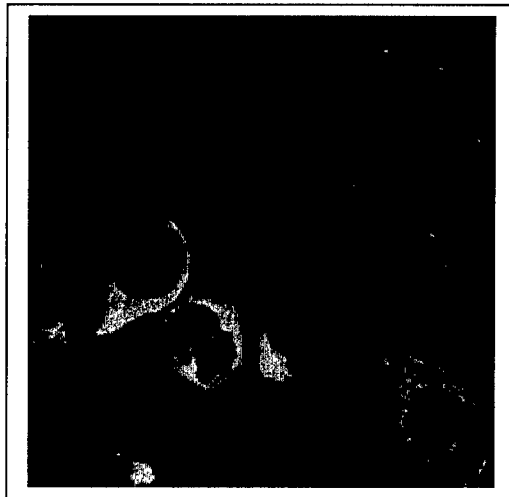


Figure 3. Immunohistochemical detection of SHBG in MCF-7 SHBG-myc23-2 cells.

Cells were plated on glass slides, fixed, and exposed to a polyclonal antibody (WAK-S102-12-53) to SHBG and developed with a donkey anti-rabbit IgG1 linked to the green fluor, Alexa -488. Nuclei were stained with the blue dye, DAPI. Shown is a representative 1 micron confocal section. Note, the staining of these overexpressing cells for SHBG-myc is much more intense than the above staining for endogenous SHBG.

Figure 4. SHBG is alternatively spliced: RT-PCR analysis of MCF-7 cells.

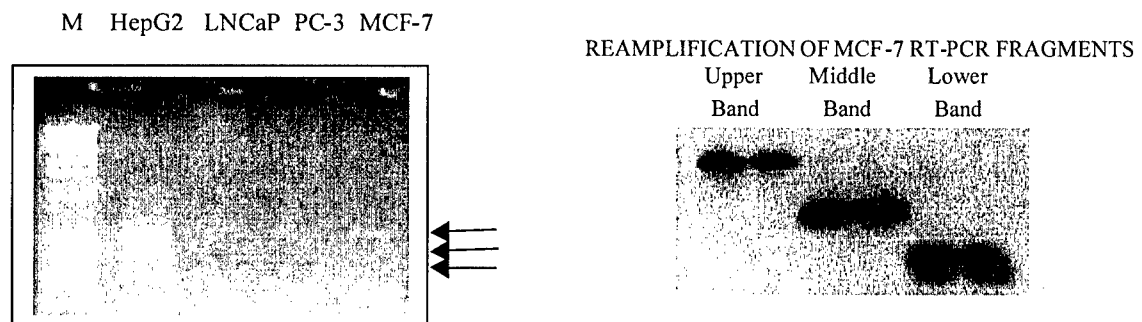


Figure 4. RT-PCR analysis reveals alternative splicing in MCF-7 cells. Left: Total cellular mRNA was prepared from exponentially growing MCF-7, HepG2 (a human hepatic cell line that secretes SHBG and was included as a positive control), LNCaP, and PC3 cells. First strand cDNAs were generated using an oligo dT primer. PCR was then performed using primers predicted to amplify exon 5-8 sequences. Three RT-PCR transcripts were generated in all samples, the predicted 521nt band, and two smaller bands (three light MCF-7 bands (arrows) were visible in the original gel). We have reproduced this alternative splicing pattern three times, using different MCF-7 total cellular RNA preparations. Right: MCF-7 RT-PCR products were electrophoresed alone in a single gel (not shown). DNA from each MCF-7 band was reamplified using the same exon 5 and 8 primers. Duplicate lanes were run for each sample (right), and DNA was extracted from the gel. DNA sequence analysis showed the 521nt band to contain contiguous exon 5-8 sequences. The middle band is missing all of exon 7, and the small band is missing exons 6 and 7. M: DNA size marker.

Figure 5. MCF-7 cell SHBG mRNAs lack hepatically secreted isoform exon 1.

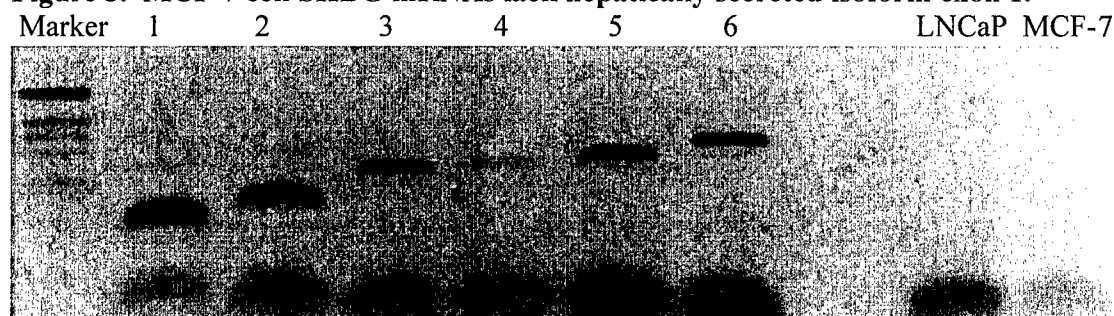


Figure 5. RT-PCR analysis of SHBG exon 1 sequences. The same first strand cDNA samples from Figure 4 were amplified by PCR. Control HepG2 RT-PCR amplifications were performed using a single exon 1 forward primer and six different exon 2 (lanes 1 and 2) or exon 3 (lanes 3-6) reverse primers in order of increasing fragment size. LNCaP and MCF-7 RT-PCR amplifications were performed using the same exon 1 forward and a single exon 3 reverse primer (the same set as in HepG2 lane 6). RT-PCR transcripts of expected sizes are present in all HepG2 amplifications. The exon 1-3 RT-PCR fragment is absent in MCF-7 and LNCaP cells (arrow).

Figure 6. Alternative SHBG gene promoter utilization and alternative splicing of SHBG exons 4, 6, and 7 in MCF-7, HepG2, and LNCaP cells, liver and testis.

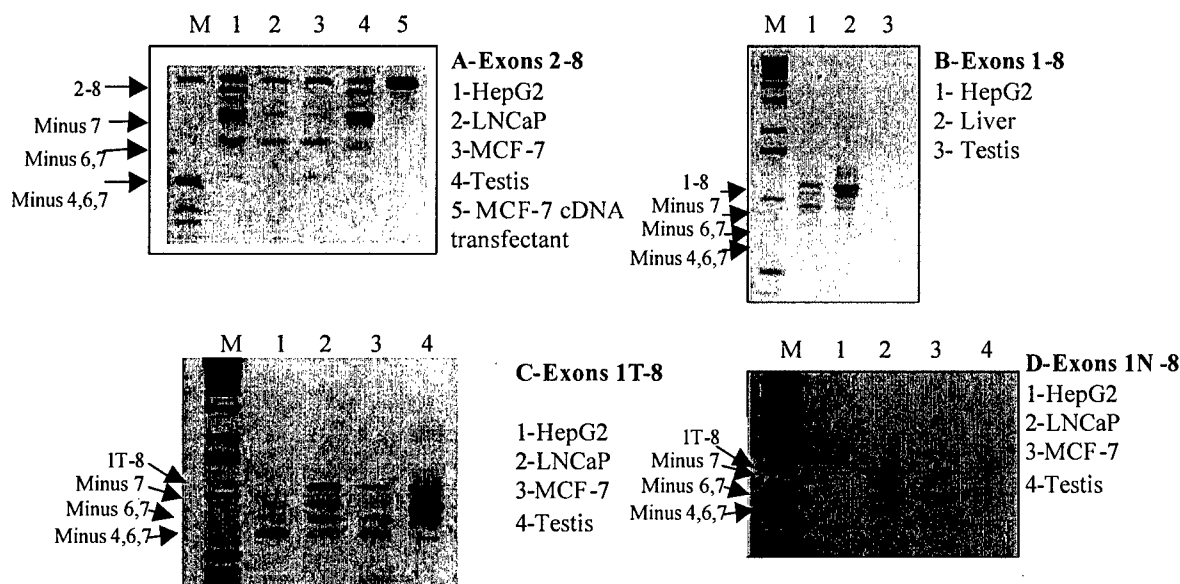
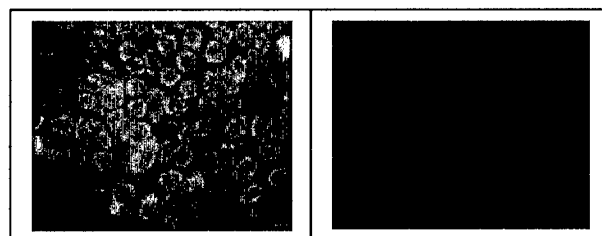


Figure 6. Expression of SHBG transcripts in human cancer cell lines, testis and liver.

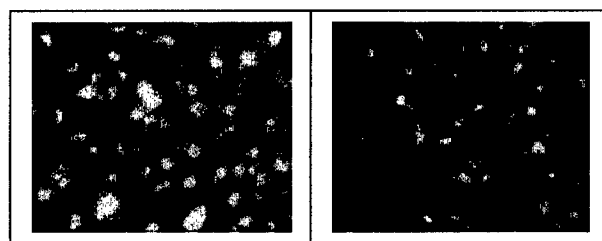
First strand oligo-dT primed cDNAs from the indicated cell lines and tissues were subject to RT-PCR amplification using primers specific for the SHBG exons shown in boldface (forward primers specific for, A- exon2, B- secreted isoform exon 1, C- testis isoform exon 1, D- novel exon 1 discovered in this study. The reverse primer for all amplifications was specific for exon 8. M- DNA Marker. Arrows point to RT-PCR fragments which have undergone sequence analysis and lack the exons denoted on the left.

Figure 7. Immunohistochemical detection of SHBG in MCF-7, MD-MBA-231, and 184-B5 cells



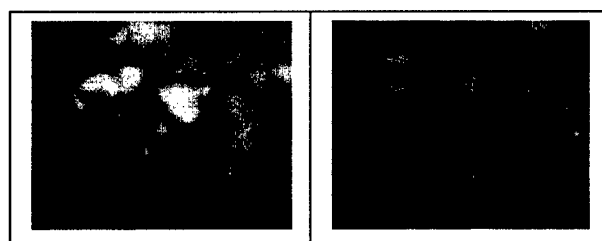
A: MCF-7 cells - SHBG

MCF-7 cells - IgG1



B: MD-MBA-231 cells - SHBG Ab

MD-MBA-231 cells - IgG1 control



C: 184-B5 cells - SHBG Ab

184-B5 cells - IgG1 control

Figure 7. Immunohistochemical detection of SHBG in MCF-7, MD-MBA-231, and 184-B5 cells. Cells were plated on glass slides, fixed, and exposed to a monoclonal antibody (5B2) to SHBG (left panels) or mouse IgG1 (right panels), and developed with a rabbit anti-mouse IgG1 linked to the green fluor, Alexa - 488. Nuclei were stained with the blue dye, DAPI.

Figure 8. SHBG binding to the MCF-7 cell surface is displaced by specific steroids

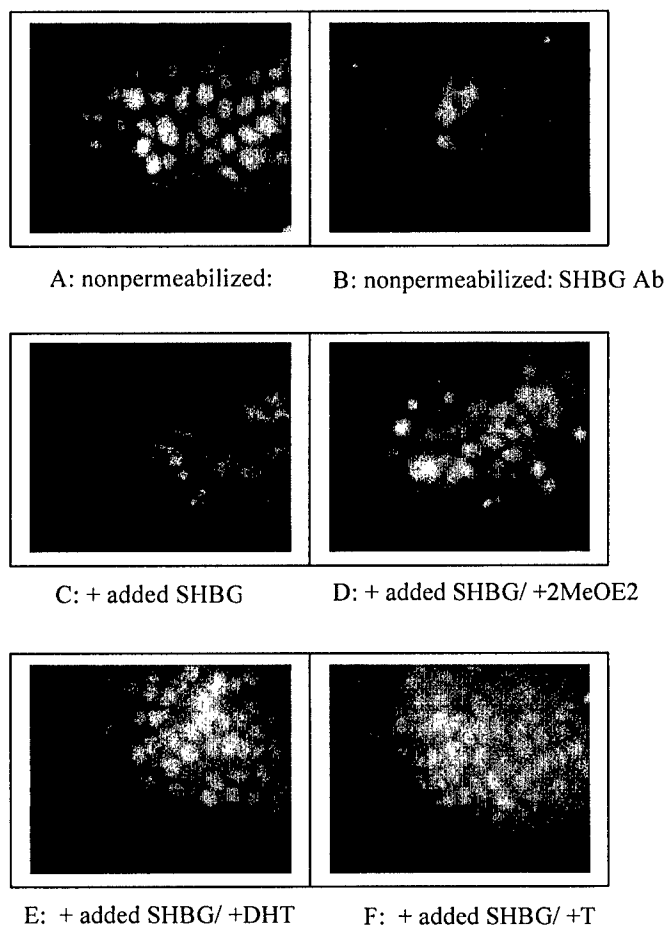


Figure 8. SHBG binding to the MCF-7 cell surface is displaced by steroids that bind to SHBG. MCF-7 cells, grown in serum free medium, were fixed and treated in the absence of the permeabilizing agent, Triton X-100. Cells were incubated in the absence of added SHBG (A, B), in the presence of added SHBG alone (C), or with SHBG and the indicated steroid (D-H). Cells were visualized with a monoclonal antibody (5B2) to SHBG (green) and were counterstained with DAPI (blue). The antibody control was mouse IgG1.

Figure 9. Immunohistochemical analysis of SHBG protein in normal breast and tumor tissue

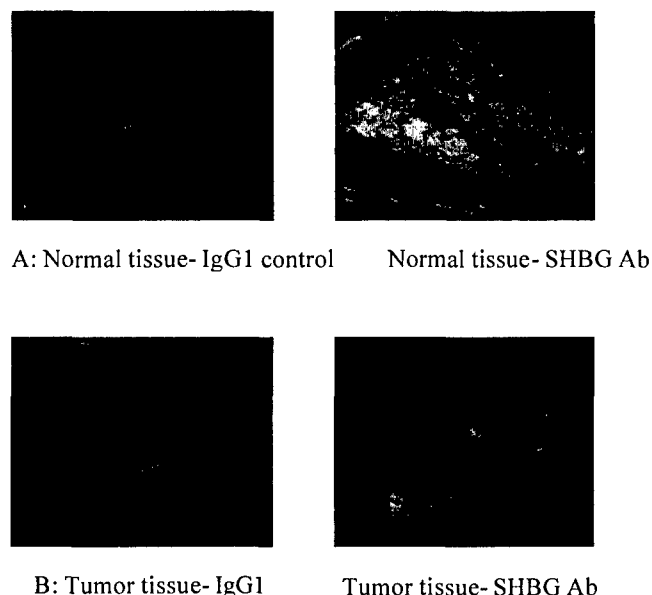


Figure 9.
Immunohistochemical
analysis of SHBG
protein in normal
breast and breast
tumors. Sections of
frozen samples of normal
human breast tissue (A),
and tumor tissue (B),
stained with either IgG1
(control-left panels), or
with rabbit anti-SHBG
(FITC, green-right
panels). Sections were
counterstained with the
nuclear stain, DAPI
(blue).

Figure 10. In situ analysis of SHBG mRNA in normal breast and tumor tissue

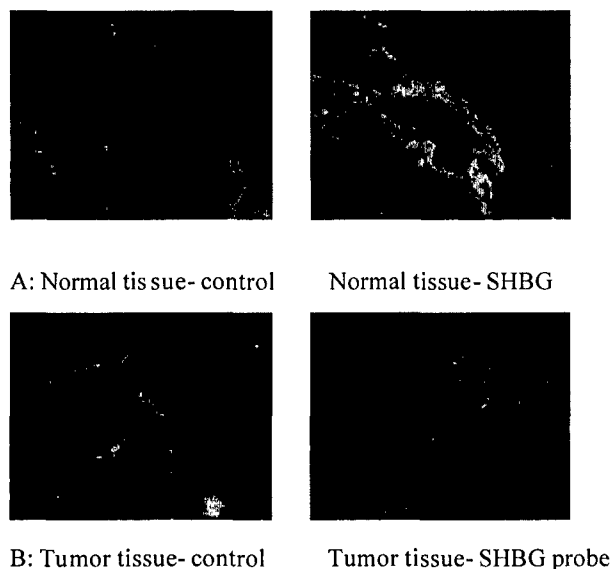


Figure 10. In Situ
hybridization of SHBG
mRNA in normal
breast and tumor
tissue. Sections were
incubated with a 521 bp
(corresponding to the
secreted SHBG cDNA
fragment from nt.628-
1148) SHBG probe.
SHBG-FITC probe,
green; nuclear
counterstain, DAPI,
blue.

Personnel

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